

Claim Objections

Claim 72 was objected to in that it did not include the word “and”. The Examiner’s objection appears correct and applicants have amended claim 72 to include to word “and” at the appropriate place.

New Matter Rejection

Claims 61, 62 and 72 were rejected under 35 U.S.C. §112, first paragraph as failing to comply with the written description requirement. Without acquiescing to the validity of the rejection applicants have deleted the terminology objected to by the Examiner. The claims have been amended to include specific language with is specifically supported in the specification as pointed out above. Further, a specific example of applicants’ invention is provided in Example 1 and the claims encompass the subject matter of Example 1.

Within the rejection the Examiner suggested that the claims be amended to indicate the lipids are complexed with the nucleic acid. The Examiner will note that new claim 72 does indicate that the particles which are comprised of the polynucleotides and polynucleotide condensing agent are complexed with the negatively charged phospholipids. This is specifically exemplified within Example 1. In that applicants have complied with the Examiner’s request and shown specific support for such complexing within Example 1 the rejection is believed to have been overcome.

Enablement Rejection

Claims 58-72 were rejected under 35 U.S.C. §112, first paragraph as not being fully enabled. Without acquiescing to the rejection the rejection is believed to have been rendered moot in view of the amendments made to the claims. Specifically, applicants have amended independent claim 72 to refer specifically to complexing the condensed polynucleotides with negatively charged phospholipids as exemplified within Example 1 and described within the specification such as at page 40, line 25 and on page 41. The rejection appears to argue that further examples of polynucleotide condensing agents should be disclosed. Applicants have disclosed such. Further, applicants specifically refer to claim 63 which lists condensing agents. In addition, see claim 64 which refers to the protamine sulfate condensing agent which is utilized in Example 1. In addition, applicants refer to dependent claims 67, 68 and 69. In addition, reference is made to the specification such as at page 10, lines 17-20 and to Example 1 and point out that this portion of the specification on page 10 refers to other publications and

at page 8 lines 4-5 indicates that all patents and publications mentioned herein are incorporated by reference. Thus, a representative number of different types of polynucleotide condensing agents are disclosed and described in the application. In view of such the rejection is believed to have been overcome.

Rejection under 35 U.S.C. §103

The claims were rejected over a variety of different references in different combinations. The rejections were traversed as applied and as they might be applied to the presently pending claims.

Within amended claim 72 applicants have indicated that the formulation is aerosolized to create particles of polynucleotides which are condensed with a polynucleotide condensing agent and that the condensed polynucleotides are complexed with a negatively charged phospholipids. Such is not taught within the cited references as taken alone or in combination with each other. It is applicants' position that the negatively charged phospholipids of Example 1 and the amended claims avoid the toxicity associated with cationic liposomes and applicants' method as now claimed is not taught within the cited references as taken alone or in combination with each other.

Applicants have attached hereto a copy of a recent publication by Patil et al. entitled "Anionic Liposomal Delivery System for DNA Transfection". This publication is dated well after the filing date of applicants application and as such is not prior art. However, the publication is submitted as third party evidence that cationic liposomes may be relatively efficient in delivering DNA to cells. However, it shows that cationic liposomes for DNA delivery can be inactivated in the presence of serum and that they may have certain instabilities during storage. Further, the Patil et al. paper points out there is cytotoxicity associated with cationic liposomes and that such effects have been shown to occur in both *in vitro* and *in vivo* situations (see Patil at page 1, col. 2 in the second full paragraph). The Patil paper states within the abstract that "transfection efficiencies of anionic lipoplexes was similar to that of cationic liposomes (lipofectAmine) whereas their toxicity was significantly lower."

First, it is applicants' position that it would not be obvious to combine the references in the manner suggested within the rejection. There are simply too many references requiring picking and choosing from different portions of each reference in different ways in order to attempt to put together an invention which even approximates applicants' invention.

Secondly, in view of the claim amendments it does not appear as though the combination of references can be made in any manner even to make a *prima facie* case of obviousness.

Thirdly, in view of the Patil et al. publication attached it is applicants position that any *prima facie* case of obviousness (which applicants do not believe has been shown) is overcome in view of the improved unexpected results obtained by using the negatively charged phospholipids as now claimed within amended claim 72, which are exemplified within Example 1 of the present application.

In view of such the 35 U.S.C. §103 rejections are believed to have been overcome and their reconsideration and withdrawal are respectfully requested.

Conclusion

The claims have been amended and support for those amendments have been pointed out above. The formal objections have been responded to. The substantive rejections have been rendered moot or overcome by the claim amendments. In view of such reconsideration and withdrawal of the rejections is respectfully requested.

In the event minor issues remain unresolved the Examiner is respectfully requested to contact the undersigned attorney at the indicated telephone number to arrange for an interview to expedite disposition of this application.

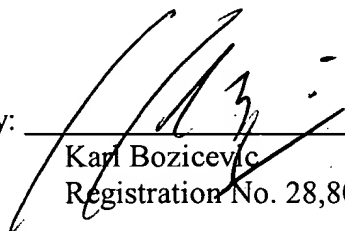
The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number AERX061.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: _____

3/Aug/05

By: _____


Karl Bozicevic
Registration No. 28,807

BOZICEVIC, FIELD & FRANCIS LLP
1900 University Avenue, Suite 200
East Palo Alto, CA 94303
Telephone: (650) 327-3400
Direct: (650) 833-7735
Facsimile: (650) 327-3231

Encl: Patil et al., "Anionic Liposomal Delivery System for DNA Transfection" *The APPS Journal*
(2004) 6(4):1-10 (article 29) (<http://www.aapsj.org>)
Notice of Recordation Reel/Frame 8379/0853 (USSN 08/752,946)
Notice of Recordation Reel/Frame 010304/0368 (USSN 09/330,903)

REMARKS

Formal Matters

Claims 58-64 and 66-73 are now pending in this application.

Claim 72 has been amended to indicate that the polynucleotides are condensed with a polynucleotide condensing agent and thereafter complexed with negatively charged phospholipids. This is supported in the specification at several places such as at page 40, lines 24-27 and within Example 1.

Claim 61 has been amended to refer to a specific phospholipids which is supported at page 41, line 22. Claim 62 has been amended to refer to a specific phospholipids which is supported at page 41, line 23. Claim 64 has been amended to include a version of a limitation contained within the earlier version of claim 72 and supported in the specification such as at page 10, lines 10-11.

Claim 66 has been amended to refer to a specific phospholipids as supported within Example 1 at page 49, line 20. New claim 73 is supported within Example 1 on page 49 at lines 10-24. No new matter has been added.

Drawing Objections

In response to the objections to the drawings applicants have attached hereto a new set of drawings which are believed to overcome each of the objections.

Priority Claim

Without acquiescing to the validity of the Examiner's position relating to priority, applicants believe the issued has been rendered moot in view of the amendments made to the claims.

Further, applicants have attached hereto a copy of an assignment filed in the present application showing that the present application is owned by Aradigm Corporation. Also attached is the assignment filed in connection with U.S. Patent 5,906,202 showing that the application is owned by Aradigm Corporation. Thus, both the current application and the U.S. Patent 5,906,202 are owned by the same entity. Further, through the undersigned attorney applicants declare that the ownership remains as indicated in the attached assignments.

Withdrawn Rejections

Applicants gratefully acknowledge the Examiner's withdrawal of the rejections under 35 U.S.C. §112, second paragraph.



Anionic Liposomal Delivery System for DNA Transfection

Submitted: January 13, 2004; Accepted: June 28, 2004; Published: October 18, 2004.

Siddhesh D. Patil,^{1,2} David G. Rhodes,¹ and Diane J. Burgess¹

¹Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269

²Current Address: Antigenics, Lexington, MA 02421

ABSTRACT

The present study investigates the use of novel anionic lipoplexes composed of physiological components for plasmid DNA delivery into mammalian cells *in vitro*. Liposomes were prepared from mixtures of endogenously occurring anionic and zwitterionic lipids, 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), respectively, at a molar ratio of 17:83 (DOPG:DOPE). Anionic lipoplexes were formed by complexation between anionic liposomes and plasmid DNA molecules encoding green fluorescence protein (GFP) using Ca^{2+} ions. Transfection and toxicity were evaluated in CHO-K1 cells using flow cytometry and propidium iodide staining, respectively. Controls included Ca^{2+} -DNA complexes (without lipids), anionic liposomes (no Ca^{2+}), and a cationic liposomal formulation. Efficient delivery of plasmid DNA and subsequent GFP expression was achieved using anionic lipoplexes. Transfection efficiency increased with Ca^{2+} concentration up to 14 mM Ca^{2+} , where transfection efficiency was 7-fold higher than in untreated cells, with minimum toxicity. Further increase in Ca^{2+} decreased transfection. Transfection efficiency of anionic lipoplexes was similar to that of cationic liposomes (lipofectAmine), whereas their toxicity was significantly lower. Ca^{2+} -DNA complexes exhibited minimal and irregular transfection with relatively high cytotoxicity. A model was developed to explain the basis of anionic lipoplex uptake and transfection efficacy. Effective transfection is explained on the formation of nonbilayer hexagonal lipid phases. Efficient and relatively safe DNA transfection using anionic lipoplexes makes them an appealing alternative to be explored for gene delivery.

KEYWORDS: anionic liposomes, gene delivery, transfection, nonviral vector, lipoplex, flow cytometry.

INTRODUCTION

Gene medicine has emerged to be one of the most potent and promising strategies for the development of cures for inherited and acquired disorders with underlying genetic

defects or malfunction.¹⁻³ More than 500 gene therapy protocols have been approved for a wide range of diseases that include cancer, cystic fibrosis, hemophilia, and neuromuscular disorders.^{4,5} Successful gene therapy necessitates targeted transfection of plasmids containing transgenes of interest into cells.⁶ Owing to the limited ability of naked DNA to enter cells and the susceptibility of DNA to enzymatic degradation, DNA transfection has largely been achieved using viral or nonviral delivery vectors.⁷ Such delivery systems can protect the DNA from degradation and have been able to successfully transfer it both *in vitro* as well as *in vivo*.^{6,8}

Nonviral transfection systems such as cationic liposomes are generally preferred over viruses because they are nonimmunogenic, are relatively easy to assemble, and are amenable to scale-up for industrial production.^{9,10} In addition, liposomal delivery vehicles offer versatility with a diverse range of morphologies, size, and release characteristics; can be used for tissue targeting; and provide protection of the plasmid DNA from attack by degradative nucleases. Since their introduction as potential delivery systems in 1987,¹¹ DNA-cationic lipid complexes, also known as cationic lipoplexes, have been used in numerous research protocols for DNA delivery in a range of cell types and are currently being investigated in several gene therapy clinical trials.¹²

However, although cationic liposomes are relatively efficient in delivering DNA into cells, they can be inactivated in the presence of serum, and there have been reports of instability upon storage.¹³ In addition, cytotoxicity of cationic liposomes remains a concern irrespective of the preferred route of DNA transfer, and such effects have been shown to occur both *in vitro*¹⁴ and *in vivo*.^{15,16} Under *in vitro* and *ex vivo* conditions, massive cell death caused by toxic delivery systems can significantly reduce their overall potential for DNA delivery. Dose-dependent pulmonary toxicity of cationic lipids due to the production of reactive oxygen intermediates has been demonstrated in mice upon intratracheal instillation of a cationic liposome preparation.¹⁵ Cationic lipids were demonstrated to be cytotoxic to phagocytic macrophages.¹⁶ The commercially available cationic lipid-based transfection agent lipofectAmine has been reported to induce toxicity in various tissues such as pulmonary cells,¹⁵ and in arterial cell walls in a porcine restenosis model for localized gene therapy.¹⁷ It is evident that there is a need for efficient and well-tolerated delivery systems to exploit the benefits of gene medicine.

Corresponding Author: Diane J. Burgess, Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269. Tel: (860) 486-3760. Fax: (860) 486-4998. Email: diane.burgess@uconn.edu.

A few studies^{18,19} that use anionic liposomal DNA delivery vectors as an alternative to cationic liposomes have been reported in recent years. Anionic liposomes have been previously used as models to simulate the cell surface to study non-receptor-mediated transport of DNA across cells.²⁰ For DNA delivery purposes, however, anionic liposomal formulations have predominantly been used to transfer oligonucleotides into hippocampal neurons¹⁹ and bacterial cells.¹⁸ However, these investigations are limited owing to (1) inefficient entrapment of DNA molecules within anionic liposomes,¹⁹ and (2) lack of toxicity data.¹⁸ Lack of further development of these systems may be attributed to the inefficient association between anionic lipids and DNA molecules resulting from the repulsive electrostatic interaction between these negatively charged species.^{21,22}

In the present study, we have investigated the potential of endogenous anionic lipids for DNA delivery and expression. This study involves the design and evaluation of an anionic liposomal delivery vector consisting of a complex between lipids of physiological origins and plasmid DNA, mediated by divalent Ca^{2+} . It was demonstrated that anionic liposomes could successfully entrap high levels of DNA by complexation with divalent Ca^{2+} ions. These lipoplexes can deliver green fluorescence protein (GFP) plasmid into Chinese hamster ovary-K1 (CHO-K1) cells with low toxicity. In addition to the uptake of a model plasmid, we have also proposed a possible mechanism for anionic lipoplex internalization and efficacy. This model is proposed based on previously reported studies that have characterized the biophysical behavior of similar mixed-composition anionic liposomes and cationic lipid-based transfection.^{21,23-32}

MATERIALS AND METHODS

Materials

Lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG), were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Calcium chloride, Luria Bertani medium, and all other reagents used for preparing buffers were purchased from Sigma Chemicals (St Louis, MO). Cell culture materials, media (Opti-MEM I Reduced Serum Medium and F12K Nutrient Mixture [Kaighn's modification]), pCMV-GFP plasmid, and lipofectAmine reagent were purchased from Invitrogen Corp (Carlsbad, CA). Hank's balanced salt solution (HBSS), used for cell culture purposes, was purchased from BioWhittaker (Walkersville, MD). CHO-K1 cell line was purchased from American Type Culture Collection (Manassas, VA).

Methods

Liposome Preparation

Liposomes were composed of a DOPG/DOPE mixture at a mol ratio of 17/83. Liposomes were prepared by reconstituting shell-dried lipid films in HEPES buffer, pH 7.4, as described previously.³² In brief, a stock solution of DOPG dissolved in chloroform was added to glass tubes. Chloroform was allowed to evaporate under a steady stream of nitrogen gas, after which the tubes were kept under vacuum to effect complete evaporation of the residual solvent. DOPG was deposited on the inner wall of the glass tubes in the form of a thin film. The actual amount of DOPG was verified gravimetrically. A stock solution of DOPE in chloroform was then added to the same tubes containing the DOPG film. The tubes were vortexed to dissolve the DOPG film in the DOPE stock solution and thus ensure homogeneous distribution of both the lipids in the tubes. The mixture of lipids was dried as explained above. The molar ratios were adjusted according to the volumes of the stock solutions. Liposomes were prepared by reconstitution of the lipid film in 10 mM HEPES buffer, pH 7.4, at 45°C, facilitated by intermittent vortexing of the suspension. The liposome suspension generated via reconstitution contained multilamellar vesicles (MLV). Small unilamellar vesicles were prepared from the MLV by sonication in an ultrasonic bath (Laboratory Supplies Co, Hicksville, NY) for 15 to 30 minutes, until the suspension achieved clarity.

Preparation of Plasmid DNA

Plasmid containing pCMV-GFP was propagated in transformed *Escherichia coli* cells. *E. coli* cells were grown in standard Luria Bertani medium at pH 7.0 to a cell density of ~ 3 to 4×10^9 cells/mL. Cells were harvested by centrifugation and the plasmid DNA was extracted and purified using a Qiagen Plasmid Maxi Kit (Qiagen, Santa Clarita, CA) as per the manufacturer's recommended protocols. In brief, the plasmid purification procedure involved alkaline hydrolysis of the cells and isolation of the plasmid by binding to an anion-exchange resin of proprietary composition. The concentration of the purified plasmid preparation was 0.87 $\mu\text{g}/\mu\text{L}$ determined using a UV-visible spectrophotometer (SpectraMax Plus, Molecular Devices, Sunnyvale, CA) using OD_{260} (optical density at wavelength 260 nm). The $\text{OD}_{260}/\text{OD}_{280}$ ratio was 1.964, indicating that the plasmid preparation was sufficiently pure and could be used for transfection purposes. Standard agarose gel electrophoresis on a 0.8% wt/wt agarose gel was conducted to investigate the plasmid structural integrity and revealed 2 major bands. The high-mobility band was attributed to the most compact or supercoiled form of plasmid DNA. The other band with low mobility indicated the overall nonsupercoil content in the plasmid preparation.

Formation of DNA-Anionic Liposome Complexes

For a single transfection experiment, 225 ng of DNA was diluted into a liposome suspension equivalent to 40 µg lipid in a centrifuge tube. Anionic lipoplex formation was achieved by addition of calcium chloride solution. Ca^{2+} ions were introduced into the formulation using stock solution of a 2 M calcium chloride solution that had been previously filter sterilized through a 0.2-µm filter. The amount of plasmid DNA was identical in all experiments irrespective of the Ca^{2+} concentrations (~7 mM to 132 mM) introduced into each complexation tube. The final volume of the lipoplex suspension applied to the cells was 300 µL. The complexes were incubated for 25 minutes at room temperature.

Particle Size Determination Studies

Anionic lipoplexes were prepared as described above and were dispersed in nanopure water. Particle size was determined using dynamic light scattering (Submicron Particle Sizer, Autodilute model 370, Particle Sizing Systems, Santa Barbara, CA). All measurements were conducted at 25°C in triplicate.

Transfection of CHO-K1 Cells

CHO-K1 cell line was cultured in complete growth medium F12K Nutrient Mixture (Kaighn's modification) containing penicillin and streptomycin supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere incubator. One day before transfection, 2.5×10^4 cells were seeded per well of a 24-well plate in 500 µL of complete growth medium, and incubated at 37°C in a 5% CO_2 environment. Cells were incubated until they were 75% to 80% confluent, which generally took 18 to 24 hours. On the day of transfection, the complete growth medium was removed from the well plates and replaced with 150 µL of transfection medium without serum (Opti-MEM 1 Reduced Serum Medium). Suspension of anionic lipoplexes, prepared as described above, was overlaid on the rinsed cells. Cells were incubated for 2 hours, after which the complex-containing suspension was removed and replaced by the original complete growth medium. For transfection experiments conducted in the presence of serum, the cells were incubated and transfected in original complete growth medium, omitting rinsing of the cells with HBSS.

For comparison of transfection efficiencies, the following controls were conducted: (1) untreated cells, (2) cells transfected with DNA, (3) cells transfected with DNA and empty anionic liposomes, (4) cells transfected with DNA and Ca^{2+} , and (5) cells transfected with cationic liposomes (lipofectAmine). Ca^{2+} -DNA complexes were prepared identically to those of anionic lipoplexes described above but without the anionic liposomes. The amount of DNA, the Ca^{2+} concentrations, and the volumes of suspension used for the transfection experi-

ments were identical to those used for anionic lipoplexes. LipofectAmine transfections were performed as per the manufacturers' instructions using 1 to 3 µL of lipofectAmine and identical amount of DNA (225 ng).

Flow Cytometry

Cells were assayed for GFP expression 48 hours after exposure to the transfection agents. In brief, cells in the well plates were rinsed with HBSS, treated using 100 µL trypsin EDTA, and incubated for 10 minutes (37°C) in a 5% CO_2 incubator to detach the adherent cells from the well plates. The cell suspension was then diluted to 1 mL using phosphate buffered saline (PBS), pH 7.4, containing 50 µL of the complete growth medium. The cells were then assayed for expression of GFP.

GFP expression was analyzed using a FACS Calibur dual laser flow cytometer (Becton-Dickinson, San Jose, CA). In addition to green fluorescence detection, forward angle light scattering (FSC) and 90° light scattering (side, SSC) measurements were conducted. GFP intensity (FL1) was determined by gating cells at an excitation wavelength of 488 nm using an argon ion laser with a standard bandpass filter. The presence of GFP was detected by emission at a wavelength of 508 nm. For each cell sample, 10 000 events were collected using the high-speed mode (200-300 cells/s). Cells were also visually inspected microscopically for the presence of fluorescence using a Leica DM IL fluorescence microscope (Leica, Wetzlar, Germany). Transfection efficiencies of various formulations were reported as multiples of those of untreated cells.

Toxicity Assay

The toxicity assay for the cells was based on the ability of dead cells to be stained by propidium iodide (PI). PI was used to assess cytotoxicity because of its ability to rapidly penetrate apoptotic cells followed by its binding to cellular DNA producing a characteristic fluorescence that can be detected using flow cytometry.³³ Preliminary experiments were conducted to ensure dead cells were not lost in the washing process during the trypsin-induced detachment within the time frame of toxicity evaluation of the formulation. Cell counting estimated an insignificant amount of dead necrotic cells in the washings. Prior to flow cytometric analysis, 20 µL of PI solution (100 µg/mL) was added to the cells. The excitation and emission wavelengths for PI fluorescence (FL2) were 536 nm and 617 nm, respectively. Toxicities of various formulations were reported as multiples of those of untreated cells.

Statistical Analysis

All transfection experiments were conducted in triplicate, and the results are shown as means ± SD. Statistical significance of results was analyzed using GraphPad Prism Version

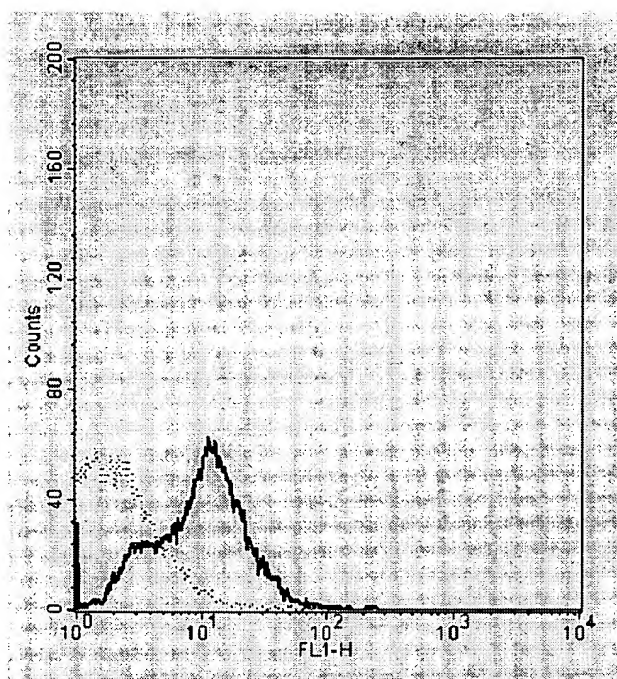


Figure 1. Representative histogram plot of flow cytometry data of CHO-K1 cells suspended in PBS, pH 7.4. The bold line represents flow cytometry pattern for transfected cells, and the dotted line represents a flow cytometry pattern for nontransfected cells. Transfections were conducted at 37°C in a 5% CO₂ environment.

4.0 for Windows (GraphPad Software, San Diego CA). Multiple comparisons were assessed using 1-way analysis of variance (ANOVA) followed by post hoc analysis using Dunnett test. Student *t* test was used to compare differences between 2 groups. Results were considered significant when probability values were < .05.

RESULTS AND DISCUSSION

Design of Anionic Lipoplexes

In this study, we have demonstrated the use of a novel anionic liposomal delivery system for transfection of mammalian CHO-K1 cells. Ca²⁺ serves as a complexing agent between plasmid DNA and the anionic liposomes. The anionic lipids used, DOPE and DOPG, are native to many biological membranes and therefore we anticipated low toxicity associated with this vector. The safety of other phosphoglycerol anionic lipids has been previously demonstrated when administered to epithelial lung tissue.¹⁵ A model plasmid containing a transgene for GFP was used as the reporter plasmid to assay the transfection efficacy of these complexes.

Particle Sizing

The average particle size of uncomplexed anionic liposomes was 114.1 ± 25.3 nm. Anionic lipoplexes formed on the addition of Ca²⁺ ions (7–68 mM Ca²⁺) had an average particle size of 464.7 ± 45.6 nm. Anionic lipoplexes obtained on the addition of higher levels of Ca²⁺ ions (>100 mM Ca²⁺) had a larger particle size of 745.4 ± 146.9 nm. Some turbidity was observed in formulations containing high levels of Ca²⁺ ions (>100 mM Ca²⁺). These larger liposomes were less effective in transfection as shown below.

tion of Ca²⁺ ions (7–68 mM Ca²⁺) had an average particle size of 464.7 ± 45.6 nm. Anionic lipoplexes obtained on the addition of higher levels of Ca²⁺ ions (>100 mM Ca²⁺) had a larger particle size of 745.4 ± 146.9 nm. Some turbidity was observed in formulations containing high levels of Ca²⁺ ions (>100 mM Ca²⁺). These larger liposomes were less effective in transfection as shown below.

Representative Flow Cytometry Data and Interpretation

Flow cytometry performed on CHO-K1 cells posttransfection demonstrated control cells and those treated with the delivery systems grew homogeneously with uniform complexity. The dotted line in Figure 1 is a typical histogram representation of flow cytometry data obtained from untreated CHO-K1 cells, whereas the solid line is representative of cells that had significant GFP expression such as those transfected with anionic lipoplexes or lipofectAmine. Untreated CHO-K1 cells possessed some innate fluorescence that is typically observed and attributed to the presence of riboflavin or flavoproteins in the cells. A shift in the fluorescence intensities of the cells was observed following successful transfection and expression of GFP. Fluorescence intensity of the majority of cells transfected using anionic lipoplexes was at least 10 times greater than that of the control cells. Cells with these or higher fluorescence intensities were used in determining the transfection efficiencies of the delivery systems.

Transfection Efficiency of Anionic Lipoplexes

Transfections were performed under identical conditions to determine the transfection efficiencies of anionic lipoplexes, their components, and lipofectAmine, using untreated cells as controls. As expected, DNA by itself and a mixture of DNA and anionic liposomes (without Ca²⁺) were incapable of transfection (Figure 2). Under identical concentrations of DNA (225 ng), Ca²⁺ (14 mM), and anionic liposomes (40 µg lipids), the transfection efficiencies of Ca²⁺-complexed DNA was negligible, resulting in fluorescence comparable with that of untreated cells in control experiments (Figure 2). In contrast, cells treated with DNA in anionic lipoplexes demonstrated high transfection efficiency, ~7-fold greater than using Ca²⁺ alone (*P* < .01) (Figure 2). Transfection efficiency of anionic lipoplexes was similar to that of the positive control lipofectAmine, which produced a ~6-fold increase in transfection (Figure 2).

Effect of Ca²⁺ on the Transfection Efficiency of Anionic Lipoplexes

In cell populations with an initial seeding density of 2.5 × 10⁴ cells per well, the delivery system was capable of plasmid DNA delivery into cells with high transfection efficiency (Figure 3). Increase in the concentration of Ca²⁺ improved the

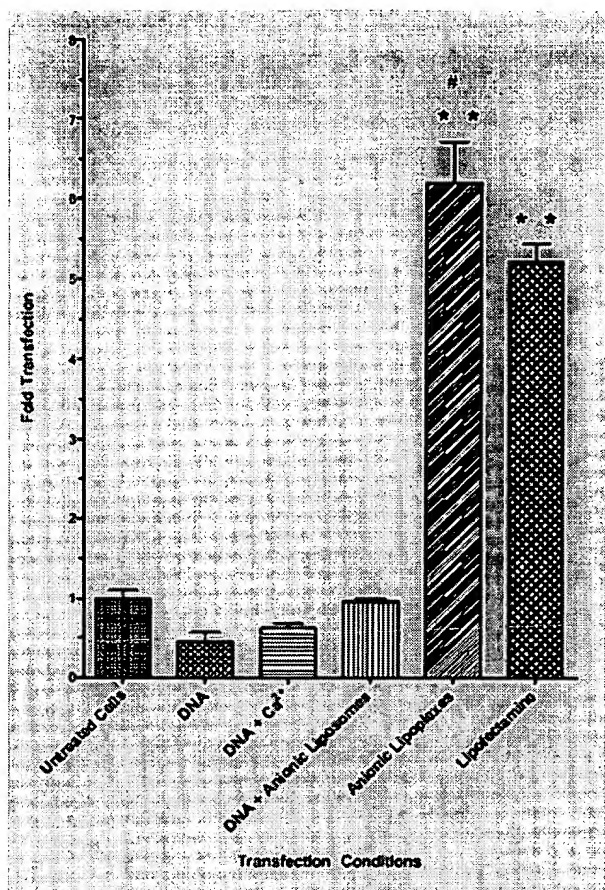


Figure 2. Comparison of transfection efficiencies of naked DNA, Ca²⁺-DNA complexes, DNA and anionic liposomes, anionic lipoplexes, and lipofectAmine along with a control of untreated cells, in identical cell populations. Transfections were conducted in serum-free medium at 37°C in a 5% CO₂ environment. For each transfection vehicle, identical amounts of DNA (225 ng) were used along with 14 mM Ca²⁺, and anionic liposomes (equivalent to 40 µg lipid), as specified. Anionic liposomes were composed of a DOPG/DOPE mixture at a mol ratio of 17:83. LipofectAmine (1 µL) was used as per manufacturer's instructions. Values represent the average of triplicates ± SD. Statistical analysis for comparing various delivery systems with control was performed using 1-way ANOVA followed by post hoc analysis using Dunnett test, * * *P* < .01. Statistical analysis for comparing anionic lipoplexes vs Ca²⁺-DNA complexes was performed using Student *t* test, # *P* = .0035.

transfection efficiency of anionic lipoplexes at least 4 times and reached a maximum of ~7-fold using 14 mM Ca²⁺ in the formulation. Any further increase in Ca²⁺ reduced the transfection efficiency but was ~4-fold higher than that of controls. The differences in the transfection efficiencies of anionic lipoplexes prepared with varying amounts of Ca²⁺ can be explained based on the mechanism of lipoplex formation (Figure 3). Since anionic lipoplexes form by interaction of anionic residues on the surface of liposomes with the phos-

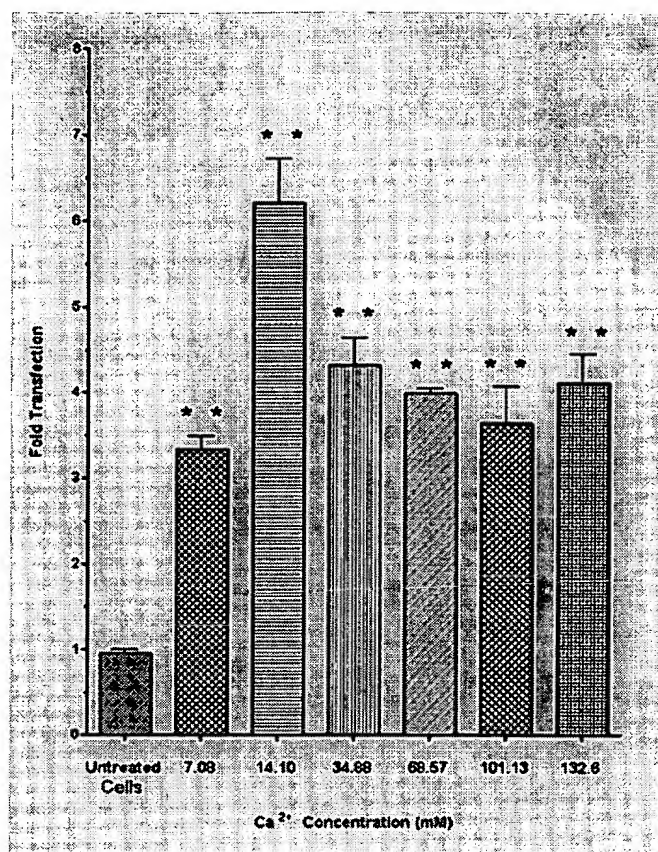


Figure 3. Effect of Ca²⁺ concentration on transfection efficiencies of anionic lipoplexes. Transfections were conducted in serum-free medium at 37°C in a 5% CO₂ environment. Values represent the average of triplicates ± SD. Statistical analysis for comparing various Ca²⁺ concentrations with control was performed using 1-way ANOVA followed by post hoc analysis using Dunnett test, * * *P* < .01.

phates in the plasmid DNA backbone, mediated by divalent Ca²⁺ bridges, increase in Ca²⁺ can increase the amount of DNA complexed to the liposomes and is predicted to increase transfection efficiencies, as was observed for Ca²⁺ concentrations up to 14 mM (Figure 3). However, stoichiometric calculations predict that there are a limited number of sites available for interaction between DNA and liposomes, thereby limiting the DNA that can be complexed to the liposome. Although the Ca²⁺ is in excess even at the optimal transfection conditions, this excess Ca²⁺ may interact with the lipoplex externally and/or associate with the DNA. These ions may associate with the lipoplex surface and be responsible for its interaction with the cell surface, thus facilitating cellular uptake.

As we showed in a previous study,³⁴ very high levels of Ca²⁺ may result in interliposome interactions leading to aggregation and increase in particle size. This increase in size and potential change in the morphology and electronic charge of the lipoplexes may be some of the contributing factors that

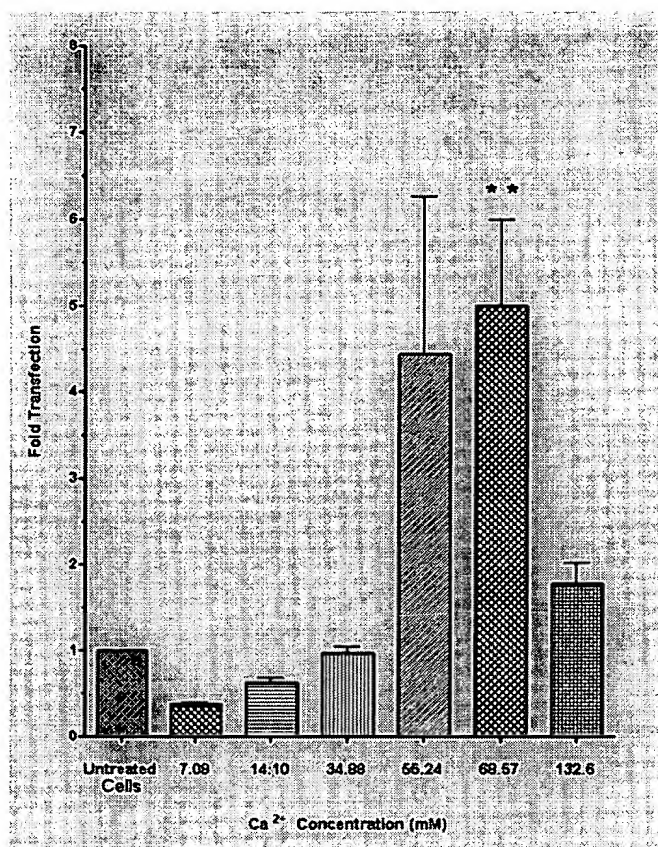


Figure 4. Effect of Ca²⁺ concentration on transfection efficiencies of DNA in the absence of anionic liposomes. Transfections were conducted in serum-free medium at 37°C in a 5% CO₂ environment. Values represent the average of triplicates \pm SD. Statistical analysis for comparing various Ca²⁺ concentrations with control was performed using 1-way ANOVA followed by post hoc analysis using Dunnet test, * * $P < .05$.

can explain the reduced transfection efficiency of anionic lipoplexes prepared with high levels of Ca²⁺, as was observed (Figure 3). This hypothesis was corroborated by measurement of lipoplex particle size at varying Ca²⁺ concentrations. We observed that anionic lipoplexes formed with low levels of Ca²⁺ with average size of 464.7 ± 45.6 nm had high transfection efficiencies, whereas those formed with higher levels of Ca²⁺ with average particle sizes of 745.4 ± 146.9 nm had lower levels of transfection. These studies are in agreement with several recently reported investigations that have shown the ability of a wide range of particles (100 nm–2 μ m) to be capable of transfection.^{35–37}

As an important control, the transfection efficiency of DNA–Ca²⁺ complexes (without the lipids) was evaluated under identical conditions and concentrations of Ca²⁺ to those used in the anionic lipoplexes since Ca²⁺ alone is capable of transfection (Figure 4). In control experiments without anionic

lipids, it was observed that low levels of Ca²⁺ (7.08, 14.10, and 55.24 mM) were incapable of transfection. However, at high levels of Ca²⁺, CHO-K1 cells were transfected, albeit very inconsistently and unreliably (Figure 4). This transfection effect was optimal at ~55 to 68 mM Ca²⁺, where transfection levels were ~5-fold. Any further increase in Ca²⁺ concentration resulted in negligible transfection (Figure 4). The Ca²⁺-mediated transfection of DNA molecules is speculated to be similar to the well-known calcium phosphate method of introducing eukaryotic DNA into mammalian cells. Ca²⁺-DNA complexes are DNA agglomerates that are formed in a nonuniform, uncontrolled, and unpredictable manner. The lack of formulator control on the production of these complexes yields particles with such inconsistent electrochemical properties as flocculate size and charge, which in turn lead to irreproducible and highly variable transfection and toxicity.

Effect of Serum on the Transfection Efficiency of Anionic Lipoplexes

As has been commonly observed with artificial DNA delivery vectors and cationic liposomes, the presence of serum reduced the transfection efficiency of anionic lipoplexes (Figure 5). Maximum transfection efficiency of formulations containing ~14 mM Ca²⁺ was reduced from ~6-fold to ~3-fold on addition of 10% fetal bovine serum to the medium (see Figure 3 for comparison). It was observed that although Ca²⁺-DNA complexes with high levels of Ca²⁺ were capable of transfection, the process was extremely low in the presence of serum and was associated with toxicity. The role of serum as a modulator of the efficacy of cationic delivery systems is extremely complex and not yet completely understood.³⁸ As implicated previously for cationic liposomes, the presence of a large fraction of DOPE in the anionic lipoplexes might have contributed to their serum inactivation.^{35,37,38} Further in vivo studies, which typically require larger amounts of DNA, have to be conducted to ascertain if serum-based inactivation indeed affects the performance of the delivery system. However, this lipoplex-mediated DNA delivery system can be used for ex vivo conditions where DNA transfer can take place in the absence of serum.

Comparison of Cytotoxicities of Various Delivery Systems

Cells that had experienced transfection either by calcium chloride, anionic lipoplexes, or by cationic lipofectAmine demonstrated higher populations of dead cells, compared with other controls, suggesting that cells that underwent transfection were vulnerable to cell death (Figure 6). Low populations of nonviable cells in control untreated cultures may be due to normal cell death during cell culture maintenance (Figure 6). As expected, anionic liposomes along with plasmid DNA showed no cytotoxicity, which was attributed primarily to their inability to interact with cell membranes

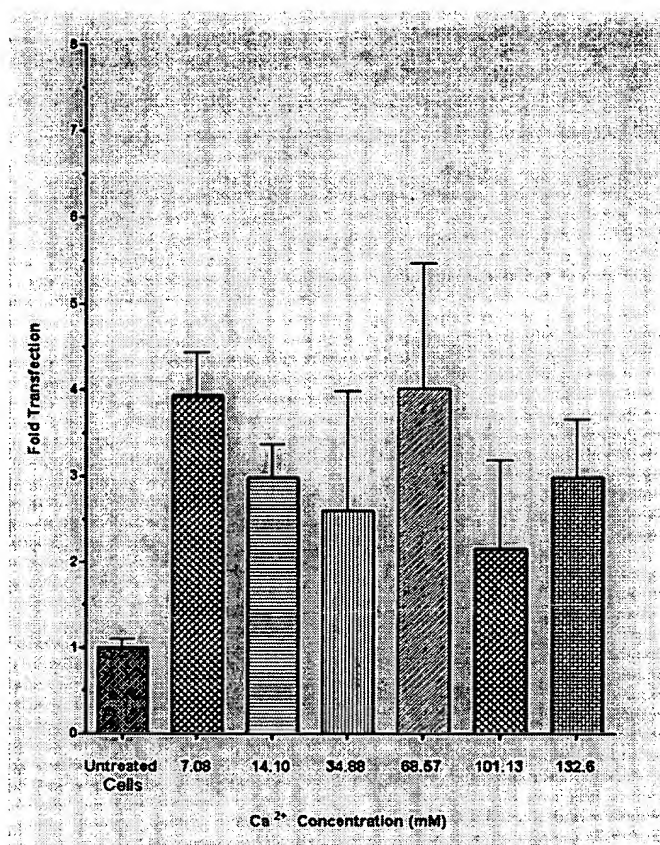


Figure 5. Effect of serum on the transfection efficiencies of anionic lipoplexes. Transfections were conducted at 37°C in a 5% CO₂ environment in the presence of 10% fetal bovine serum in the medium. Values represent the average of triplicates \pm SD.

(Figure 6). On the contrary, cationic lipids have been shown to be cytotoxic even when administered on their own.¹⁵

The toxicity of anionic lipoplexes and Ca²⁺-DNA complexes directly correlated to the transfection efficiency of the formulations (Figures 3, 4, 7, and 8) and was dependent on the concentration of Ca²⁺ in the formulation. For anionic lipoplexes, there was a peak in toxicity at ~14 mM Ca²⁺ (~3-fold), but toxicity decreased to less than 2-fold at Ca²⁺ concentrations \geq ~35 mM (Figure 7). Ca²⁺-DNA complexes produced high toxicity with very high variability. Ca²⁺-DNA complexes formulated with low levels of Ca²⁺ (\leq 34.88 mM) exhibited negligible toxicity, whereas higher concentrations of Ca²⁺, (such as ~55 and 68 mM) demonstrated significant cell toxicity (3- to 4-fold higher than controls), with high variability (Figure 8). Toxic effects of Ca²⁺-DNA complexes prepared with high levels of Ca²⁺ may be attributed to the presence of Ca²⁺, based on the high levels of toxicity observed when CHO-K1 cells were independently treated with calcium chloride. Other studies have also demonstrated that Ca²⁺ can cause phospholipid reorganization, which may be responsible for membrane destabi-

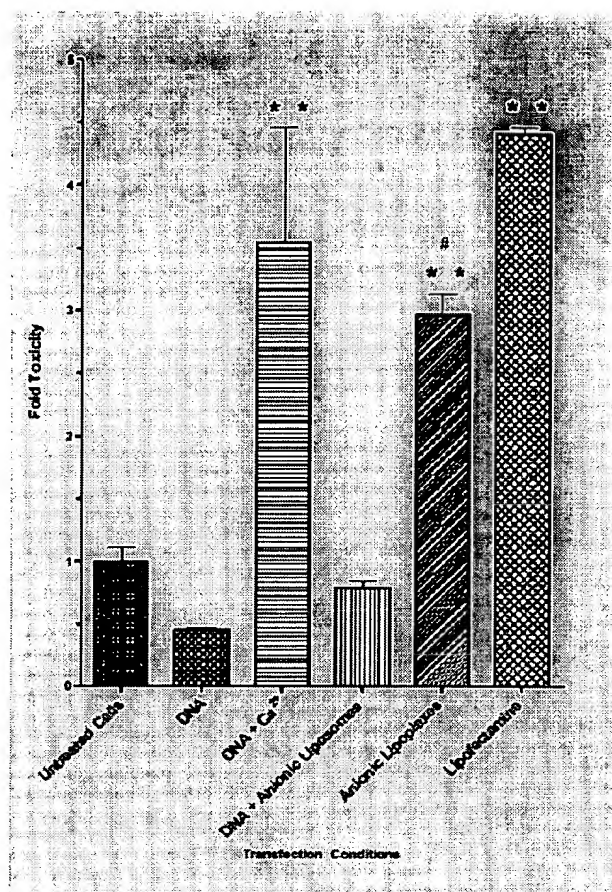


Figure 6. Comparison of cytotoxicities of naked DNA, Ca²⁺-DNA complexes, DNA and anionic liposomes, anionic lipoplexes, and lipofectAmine along with a control of untreated cells, in identical cell populations. Transfections were conducted with identical amounts of DNA and Ca²⁺ in 2.5×10^4 cells, in serum-free medium at 37°C in a 5% CO₂ environment. Anionic liposomes were composed of a DOPG/DOPE mixture at a mol ratio of 17/83. LipofectAmine (1 μ L) was used as per manufacturer's instructions. Values represent the average of triplicates \pm SD. Statistical analysis for comparing various delivery systems with control was performed using 1-way ANOVA followed by post hoc analysis using Dunnet test, * * $P < .01$. Statistical analysis for comparing anionic lipoplexes vs lipofectAmine was performed using Student *t* test, # $P = .0073$.

lization.^{30,31} Although the cationic lipoplex formulation, lipofectAmine, had comparable transfection efficiency with that of anionic lipoplexes, toxicity was 40% higher for lipofectAmine compared with the anionic lipoplexes. It should also be noted that for identical cell populations and DNA content, anionic lipoplexes, even at ~10-fold higher doses, had 40% lower toxicity than cationic liposomes. It is evident that complexes made from anionic lipids are safer and better tolerated compared with lipofectAmine and Ca²⁺-DNA complexes.

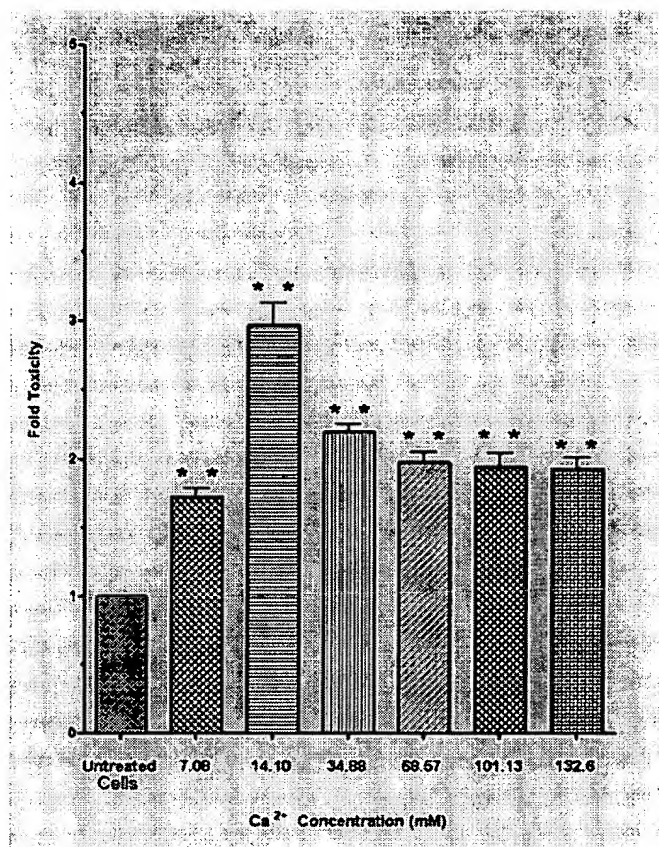


Figure 7. Effect of Ca^{2+} concentration on toxicities of anionic lipoplexes. Transfections were conducted in serum-free medium at 37°C in a 5% CO_2 environment. Values represent the average of triplicates \pm SD. Statistical analysis for comparing Ca^{2+} concentrations to control was performed using 1-way ANOVA followed by post hoc analysis using Dunnet test, * $P < .01$ and * $P < .05$.

Model for Mechanism of Anionic Lipoplex-mediated DNA Transfection

The mechanism of anionic lipoplex-mediated transfection may be speculated based on (1) our previous investigations on the biophysical interaction of anionic liposomes with oligonucleotides as well as plasmid DNA in the presence of divalent cations (S.D.P., D.G.R., D.J.B., unpublished data, 2004),^{21,32} (2) the phase behavior of mixed composition liposomes of unsaturated zwitterionic DOPE and anionic DOPG lipids in the presence of Ca^{2+} ,^{26, 29-31} and (3) previously established models of cationic lipid-based transfection.^{23-25,27,28}

We have previously demonstrated that interaction of plasmid DNA, Ca^{2+} , and anionic liposomes during the formation of ternary anionic lipoplexes is dramatically different from that of the binary complexes of plasmid DNA and Ca^{2+} alone (S.D.P., D.G.R., D.J.B., unpublished data, 2004). The formation of anionic lipoplexes induced a conformational transi-

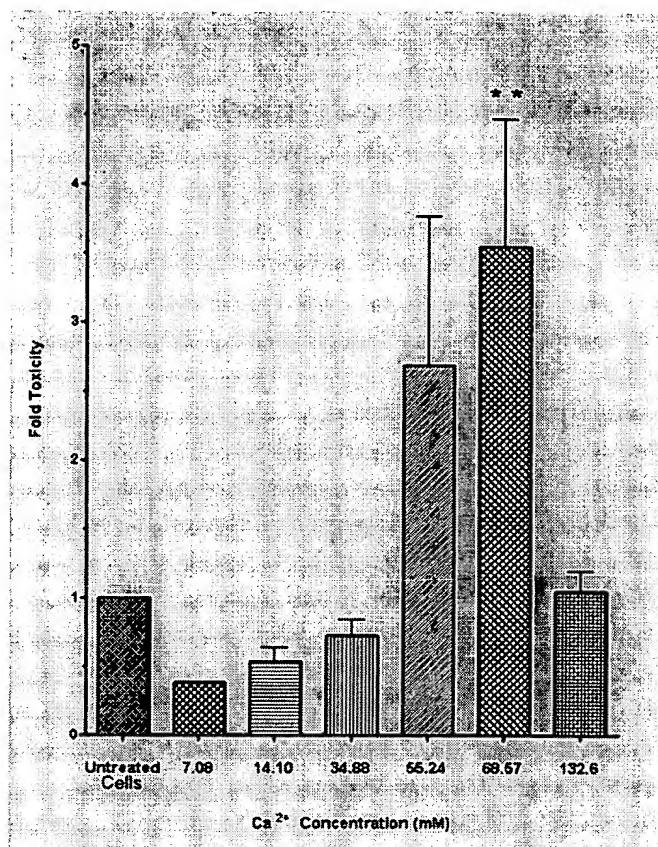


Figure 8. Effect of Ca^{2+} concentration on toxicities of Ca^{2+} -DNA complexes in the absence of anionic liposomes. Transfections were conducted in serum-free medium at 37°C in a 5% CO_2 environment. Values represent the average of triplicates \pm SD. Statistical analysis for comparing Ca^{2+} concentrations to control was performed using 1-way ANOVA followed by post hoc analysis using Dunnet test, * $P < .05$.

tion from the native B-DNA conformation of the plasmid into the compact and condensed Z-DNA; Ca^{2+} alone, even at concentrations 1000-fold higher, could not facilitate this transformation. The electrophoretic mobility of anionic lipoplexes was retarded due to higher particle size, whereas Ca^{2+} -DNA complexes had no effect on the mobility of DNA.

Cellular internalization of cationic lipoplexes and mechanism of DNA release from cationic lipoplexes can be explained based on the model put forth by the Szoka and coworkers.^{25,27,28} This model suggests that cellular uptake of cationic lipoplexes first results in their accumulation in endosomes followed by intracellular DNA release due to destabilization of the endosomal membrane. Interaction of endosomal membrane anionic lipids with the cationic lipoplexes through formation of ion pairs, lateral diffusion, and lipid mixing are some of the mechanisms speculated to facilitate the DNA and lipoplex release.

X-ray diffraction studies have also indicated that cationic lipoplexes are successful in transfection because of the formation of the H_{II}^c (hexagonal) phase instead of the $L\alpha$ (lamellar) phase due to the overall molecular inverted-cone shape of the lipid and the small, less-hydrated DOPE head group in the lipoplexes.^{23,25} In addition, fluorescent cationic DOPE-containing lipoplexes in the H_{II}^c phase have been demonstrated to undergo rapid fusion and release DNA intracellularly.²⁴

On the contrary, in the case of mixed composition anionic liposomes with sufficient levels of DOPG similar to those used in this study, metastable $L\alpha$ -phase bilayers have been shown to spontaneously occur instead of the transfection-favoring non-bilayer reversed hexagonal H_{II} configuration.²⁵ In addition, strong electrostatic repulsion between the liposomes and plasmid DNA normally prevents any association of the 2 components. However, during anionic lipoplex formation, addition of Ca^{2+} not only stimulates binding of DNA to the liposome (S.D.P., D.G.R., D.J.B., unpublished data, 2004), but also has been shown to promote and stabilize the formation of the H_{II} phase, which, as in the case of cationic liposomes, is hypothesized to facilitate cellular uptake.²⁶

In the case of anionic lipoplexes, upon endosomal compartmentalization, the formation of lipid ion pairs that promote release of the lipoplex is impossible because lipid populations in both the endosomal membrane lipids and those in the anionic lipoplex have negative charges. Previous studies have demonstrated that Ca^{2+} interaction may cause lipid scrambling,²⁹ and intercellular Ca^{2+} ions may induce redistribution of the phospholipids in erythrocyte membranes.^{30,31} Based on these reports, it is speculated that such mechanisms as lipid scrambling and redistribution caused due Ca^{2+} ions dissociated from the anionic lipoplexes may play a role in endosome destabilization and release of DNA from anionic lipoplexes. Additional biophysical experimentation needs to be conducted to conclusively demonstrate such phenomena.

CONCLUSION

Efficient delivery of plasmid DNA was achieved in mammalian cell cultures using anionic lipoplexes. Anionic liposomes by themselves, as expected, did not transfect the mammalian cells owing to the lack of interaction between similarly charged DNA and lipids. However anionic lipoplexes were capable of DNA delivery. Although Ca^{2+} alone is capable of facilitating DNA transfection, Ca^{2+} interaction with DNA results in precipitation and, consequently, transfection is highly variable. Toxicity of naturally occurring anionic lipoplexes is demonstrated to be lower than the cationic liposome lipofectamine and Ca^{2+} -DNA complexes. A mechanism of anionic lipoplex transfection is proposed on the basis of the formation of nonbilayer hexagonal phases in the lipoplex that facilitate cellular internalization. Anionic lipoplexes described here might be useful in ex vivo and in

vitro applications for DNA transfer particularly in cell lines sensitive to the toxic effects of cationic lipids.

ACKNOWLEDGEMENTS

The authors would like to thank Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT), for the generous support of a predoctoral graduate fellowship for Siddhesh Patil; Dr Michelle Barber and Ms Joan Jewett, Flow Cytometry and Confocal Microscopy Facility, Biotechnology Center, University of Connecticut, Storrs, CT, for assistance with flow cytometry experiments; and Dr Brian Aneskievich, and Katalin Kermecsei, Department of Pharmaceutical Sciences, University of Connecticut, for technical assistance.

REFERENCES

1. Patil SD, Burgess DJ. DNA-based biopharmaceuticals: therapeutics for the 21st century. *AAPS NewsMagazine*. 2003;6:27.
2. Patil SD, Rhodes DG, Burgess DJ. DNA-based therapeutics and DNA delivery systems: a comprehensive review. *AAPS PharmSci*. 2004; In press.
3. Romano G, Micheli P, Pacilio C, Giordano A. Latest developments in gene transfer technology: achievements, perspectives, and controversies over therapeutic applications. *Stem Cells*. 2000;18(1):19-39.
4. Vorburger SA, Hunt KK. Adenoviral gene therapy. *Oncologist*. 2002;7:46-59.
5. Eastman SJ, Scheule RK. Cationic lipid:pDNA complexes for the treatment of cystic fibrosis. *Curr Opin Mol Ther*. 1999;1:186-196.
6. Luo D, Saltzman WM. Synthetic DNA delivery systems. *Nat Biotechnol*. 2000;18:33-37.
7. Liu F, Huang L. Development of non-viral vectors for systemic gene delivery. *J Control Release*. 2002;78(1-3):259-266.
8. Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med*. 2001;7:33-40.
9. Zhdanov RI, Podobed OV, Vlassov VV. Cationic lipid-DNA complexes—lipoplexes—for gene transfer and therapy. *Bioelectrochemistry*. 2002;58:53-64.
10. Brown MD, Schatzlein AG, Uchegbu IF. Gene delivery with synthetic (non viral) carriers. *Int J Pharm*. 2001;229(1-2):1-21.
11. Felgner PL, Gadek TR, Holm M, et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A*. 1987;84:7413-7417.
12. Audouy SA, de Leij LF, Hoekstra D, Molema G. In vivo characteristics of cationic liposomes as delivery vectors for gene therapy. *Pharm Res*. 2002;19:1599-1605.
13. Marshall J, Yew NS, Eastman SJ, Jiang C, Scheule RK, Cheng SH. Cationic lipid-mediated gene delivery to the airways. In: Huang L, Hung M-C, Wagner E, eds. *Nonviral Vectors for Gene Therapy*. San Diego, CA: Academic Press; 1999:39-68.
14. Lappalainen K, Jaaskelainen I, Syrjanen K, Urtti A, Syrjanen S. Comparison of cell proliferation and toxicity assays using two cationic liposomes. *Pharm Res*. 1994;11:1127-1131.
15. Dokka S, Toledo D, Shi X, Castranova V, Rojanasakul Y. Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes. *Pharm Res*. 2000;17:521-525.

16. Filion MC, Phillips NC. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim Biophys Acta*. 1997;1329:345-356.
17. Armeanu S, Pelisek J, Krausz E, et al. Optimization of nonviral gene transfer of vascular smooth muscle cells in vitro and in vivo. *Mol Ther*. 2000;1:366-375.
18. Fillion P, Desjardins A, Sayasith K, Lagace J. Encapsulation of DNA in negatively charged liposomes and inhibition of bacterial gene expression with fluid liposome-encapsulated antisense oligonucleotides. *Biochim Biophys Acta*. 2001;1515:44-54.
19. Lakkaraju A, Dubinsky JM, Low WC, Rahman Y-E. Neurons are protected from excitotoxic death by p53 antisense oligonucleotides delivered in anionic liposomes. *J Biol Chem*. 2001;276:32000-32007.
20. Akhtar S, Basu S, Wickstrom E, Juliano RL. Interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). *Nucleic Acids Res*. 1991;19:5551-5559.
21. Patil SD, Rhodes DG. Influence of divalent cations on the conformation of phosphorothioate oligodeoxynucleotides: a circular dichroism study. *Nucleic Acids Res*. 2000;28:2439-2445.
22. Perrie Y, Gregoriadis G. Liposome-entrapped plasmid DNA: characterization studies. *Biochim Biophys Acta*. 2000;1475:125-132.
23. Safinya CR. Structures of lipid-DNA complexes: supramolecular assembly and gene delivery. *Curr Opin Struct Biol*. 2001;11:440-448.
24. Koltover I, Salditt T, Radler JO, Safinya CR. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. *Science*. 1998;281:78-81.
25. Hope MJ, Mui B, Ansell S, Ahkong QF. Cationic lipids, phosphatidylethanolamine and the intracellular delivery of polymeric, nucleic acid-based drugs. *Mol Membr Biol*. 1998;15:1-14.
26. Tilcock CPS, Cullis PR, Gruner SM. Calcium-induced phase separation phenomena in multicomponent unsaturated lipid mixtures. *Biochemistry*. 1988;27:1415-1420.
27. Xu Y, Szoka FC Jr. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry*. 1996;35:5616-5623.
28. Szoka FC Jr, Xu Y, Zelphati O. How are nucleic acids released in cells from cationic lipid-nucleic acid complexes? *J Liposome Res*. 1996;6:567-587.
29. Sulpice J-C, Moreau C, Devaux PF, Zachowski A, Giraud F. Antagonist effects of Ca^{2+} and spermine on phosphatidylinositol 4,5-bisphosphate-mediated transmembrane redistribution of phospholipids in large unilamellar vesicles and in erythrocytes. *Biochemistry*. 1996;35:13345-13352.
30. Williamson P, Kulick A, Zachowski A, Schlegel RA, Devaux PF. Calcium induces transbilayer redistribution of all major phospholipids in human erythrocytes. *Biochemistry*. 1992;31:6355-6360.
31. Bucki R, Bachelot-Loza C, Zachowski A, Giraud F, Sulpice J-C. Calcium induces phospholipid redistribution and microvesicle release in human erythrocyte membranes by independent pathways. *Biochemistry*. 1998;37:15383-15391.
32. Patil SD, Rhodes DG. Conformation of oligodeoxynucleotides associated with anionic liposomes. *Nucleic Acids Res*. 2000;28:4125-4129.
33. Merrick P, Nieminen AL, Harper RA, Henman B, Lemasters JJ. Cytotoxicity screening of surfactant-based shampoos using a multiwell fluorescence scanner: correlation with Draize eye scores. *Toxicol In Vitro*. 1992;6:543-547.
34. Tari AM, Fuller N, Boni LT, Collins D, Rand P, Huang L. Interactions of liposome bilayers composed of 1,2-diacyl-3-succinylglycerol with protons and divalent cations. *Biochim Biophys Acta*. 1994;1192:253-262.
35. Almofti MR, Harashima H, Shinohara Y, Almofti A, Li W, Kiwada H. Lipoplex size determines lipofection efficiency with or without serum. *Mol Membr Biol*. 2003;20:35-43.
36. Ross PC, Hui SW. Lipoplex size is a major determinant of in vitro lipofection efficiency. *Gene Ther*. 1999;6:651-659.
37. Turek J, Dubertret C, Jaslin G, Antonakis K, Scherman D, Pitard B. Formulations which increase the size of lipoplexes prevent serum-associated inhibition of transfection. *J Gene Med*. 2000;2:32-40.
38. Audouy S, Molema G, De Leij L, Hoekstra D. Serum as a modulator of lipoplex-mediated gene transfection: dependence of amphiphile, cell type and complex stability. *J Gene Med*. 2000;2:465-476.

RECEIVED

MAY 13 1997

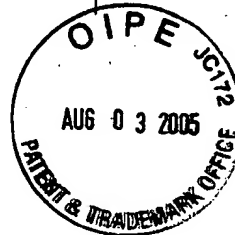
FISH & RICHARDSON P.C.

MAY 06, 1997 SILICON VALLEY OFFICE PTAS

FISH & RICHARDSON P.C.
KARL BOZICEVIC
2200 SAND HILL ROAD, SUITE 100
MENLO PARK, CA 94025



K413
UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
ASSISTANT SECRETARY AND COMMISSIONER
OF PATENTS AND TRADEMARKS
Washington, D.C. 20231



100369971A

UNITED STATES PATENT AND TRADEMARK OFFICE
NOTICE OF RECORDATION OF ASSIGNMENT DOCUMENT

THE ENCLOSED DOCUMENT HAS BEEN RECORDED BY THE ASSIGNMENT DIVISION OF THE U.S. PATENT AND TRADEMARK OFFICE. A COMPLETE MICROFILM COPY IS AVAILABLE AT THE ASSIGNMENT SEARCH ROOM ON THE REEL AND FRAME NUMBER REFERENCED BELOW.

PLEASE REVIEW ALL INFORMATION CONTAINED ON THIS NOTICE. THE INFORMATION CONTAINED ON THIS RECORDATION NOTICE REFLECTS THE DATA PRESENT IN THE PATENT AND TRADEMARK ASSIGNMENT SYSTEM. IF YOU SHOULD FIND ANY ERRORS OR HAVE QUESTIONS CONCERNING THIS NOTICE, YOU MAY CONTACT THE EMPLOYEE WHOSE NAME APPEARS ON THIS NOTICE AT 703-308-9723. PLEASE SEND REQUEST FOR CORRECTION TO: U.S. PATENT AND TRADEMARK OFFICE, ASSIGNMENT DIVISION, BOX ASSIGNMENTS, NORTH TOWER BUILDING, SUITE 10C35, WASHINGTON, D.C. 20231.

RECORDATION DATE: 03/04/1997

REEL/FRAME: 8379/0853
NUMBER OF PAGES: 3

BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

ASSIGNOR:
SCHUSTER, JEFFREY A.

DOC DATE: 02/24/1997

ASSIGNOR:
GONDA, IGOR

DOC DATE: 02/24/1997

ASSIGNEE:
ARADIGM CORPORATION
26219 EDEN LANDING ROAD
HAYWARD, CALIFORNIA 94545

SERIAL NUMBER: 08752946 ✓
PATENT NUMBER:

FILING DATE: 11/21/1996 ✓
ISSUE DATE:

PEARLENE FOSTER, EXAMINER
ASSIGNMENT DIVISION
OFFICE OF PUBLIC RECORDS

Reviewed By Franchise Systems
Initials: JVS
Reviewed By Billing Secretary
Initials: NLS

03-12-1997

Attorney Docket No.: 06513/043001

MED 3-4-97

Commissioner of Patents :



100369971

OVER SHEET

JLY

RECEIVED
al documents

ched original documents.

MAR 04 1997
and address

Name and address of receiving party(ies):

ARACORP CORPORATION
26219 Eden Landing Road
Hayward, CA 94545

1. Name of conveying party(ies).

JEFFREY A. SCHUSTER
IGOR GONDA

Additional name(s) attached? ☐ Yes ☐ No

3. Nature of conveyance:

- ☐ Assignment
☐ Merger
☐ Security Agreement
☐ Change of Name
☐ Other:

Additional names/addresses attached? ☐ Yes ☒ No

Execution Date: February 24, 1997

4. Application number(s) or patent number(s):

If this document is being filed with a new application, the execution date of the application is:

A. Patent Application No.(s):

08/752,946

B. Patent No.(s):

Additional numbers attached? ☐ Yes ☐ No

5. Name/address of party to whom correspondence concerning document should be mailed:

Karl Bozicevic
Fish & Richardson P.C.
2200 Sand Hill Road, Suite 100
Menlo Park, CA 94025

6. Total number of applications/patents involved: 1

7. Total fee (37 CFR 3.41): **\$40**

- ☐ Enclosed
☐ Authorized to charge deposit account

8. Deposit account number: **06-1050**

If the fee above is being charged to deposit account, a duplicate copy of this cover sheet is attached. Please apply any additional charges, or any credits, to our Deposit Account No. 06-1050.

DO NOT USE THIS SPACE

9. Statement and signature: *To the best of my knowledge and belief, the foregoing information is true and correct and the attached is the original document.*

Karl Bozicevic, Reg. No. 28,807

Name of Person Signing

Signature

Feb 28, 1997 Date

Date _____

Total number of pages including cover sheet, attachments, and document: 3

Date of Deposit

I hereby certify under 37 CFR 1.8(g) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

ASSIGNMENT

For valuable consideration, we, JEFFREY A. SCHUSTER, of Oakland, CA; IGOR GONDA of San Francisco, CA; hereby assign to ARADIGM CORPORATION, a CALIFORNIA corporation having a place of business at 26219 Eden Landing Road, Hayward, CA 94545, and its successors and assigns (collectively hereinafter called "the Assignee"), the entire right, title and interest throughout the world in the inventions and improvements which are subject of an application for United States Patent signed by us, entitled

DEVICE AND METHOD FOR DIRECTING AEROSOLIZED
MIST TO A SPECIFIC AREA OF THE RESPIRATORY TRACT

Filed: November 21, 1996, and

Assigned U.S. Serial No: 08/752,946

and we authorize and request the attorneys appointed in said application to hereafter complete this assignment by inserting above the filing date and serial number of said application when known; this assignment including said application, any and all United States and foreign patents, utility models, and design registrations granted for any of said inventions or improvements, and the right to claim priority based on the filing date of said application under the International Convention for the Protection of Industrial Property, the Patent Cooperation Treaty, the European Patent Convention, and all other treaties of like purposes; and we authorize the Assignee to apply in all countries in our name or in its own name for patents, utility models, and design registrations and like rights of exclusion and for inventors' certificates for said inventions and improvements; and we agree for ourselves and our respective heirs, legal representatives and assigns, without further compensation to perform such lawful acts and to sign such further applications, assignments, Preliminary Statements and other lawful documents as the Assignee may reasonably request to effectuate fully this assignment.

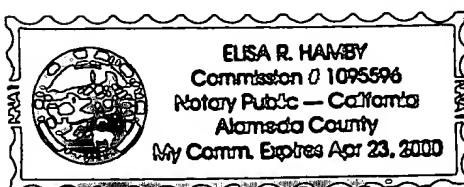

JEFFREY A. SCHUSTER L.S.

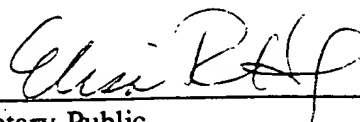
IN WITNESS WHEREOF, I hereto set my hand and seal at Hayward, CA,
this 24th day of February, 1997

STATE OF California:
COUNTY OF Alameda: ss.

Before me this 24th day of February, 1997, personally appeared

Jeffrey A. Schuster known to me to be the person whose name is subscribed to the foregoing Assignment, and acknowledged that he/~~she~~ executed the same as his/~~her~~ free act and deed for the purposes therein contained.




Notary Public
My Commission Expires: 4-23-00

[Notary's Seal Here]

Igor Gonda
IGOR GONDA

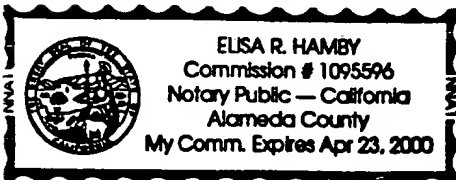
L.S.

IN WITNESS WHEREOF, I hereto set my hand and seal at Hayward, CA,
this 24th day of February, 1997

STATE OF California :
COUNTY OF Alameda : ss.

Before me this 24th day of February, 1997, personally appeared

Igor Gonda known to me to be the person whose name is subscribed to the
foregoing Assignment, and acknowledged that he/~~she~~ executed the same as his/~~her~~ free act and deed for
the purposes therein contained.



[Notary's Seal Here]

Elisa R. Hamby
Notary Public
My Commission Expires: 4-23-00

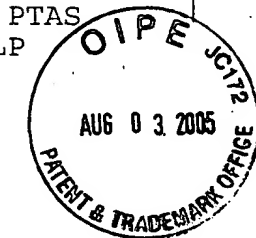
JAN 19 2000



1 AERX-021
UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
ASSISTANT SECRETARY AND COMMISSIONER
OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

JANUARY 10, 2000

BOZICEVIC, FIELD, & FRANCIS LLP
KARL BOZICEVIC
285 HAMILTON AVENUE
SUITE 200
PALO ALTO, CA 94301



101173261A

UNITED STATES PATENT AND TRADEMARK OFFICE
NOTICE OF RECORDATION OF ASSIGNMENT DOCUMENT

THE ENCLOSED DOCUMENT HAS BEEN RECORDED BY THE ASSIGNMENT DIVISION OF THE U.S. PATENT AND TRADEMARK OFFICE. A COMPLETE MICROFILM COPY IS AVAILABLE AT THE ASSIGNMENT SEARCH ROOM ON THE REEL AND FRAME NUMBER REFERENCED BELOW.

10
PAP
PLEASE REVIEW ALL INFORMATION CONTAINED ON THIS NOTICE. THE INFORMATION CONTAINED ON THIS RECORDATION NOTICE REFLECTS THE DATA PRESENT IN THE PATENT AND TRADEMARK ASSIGNMENT SYSTEM. IF YOU SHOULD FIND ANY ERRORS OR HAVE QUESTIONS CONCERNING THIS NOTICE, YOU MAY CONTACT THE EMPLOYEE WHOSE NAME APPEARS ON THIS NOTICE AT 703-308-9723. PLEASE SEND REQUEST FOR CORRECTION TO: U.S. PATENT AND TRADEMARK OFFICE, ASSIGNMENT DIVISION, BOX ASSIGNMENTS, CG-4, 1213 JEFFERSON DAVIS HWY, SUITE 320, WASHINGTON, D.C. 20231.

RECORDATION DATE: 10/12/1999

REEL/FRAME: 010304/0368
NUMBER OF PAGES: 3

BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

ASSIGNOR:

✓ GONDA, IGOR

✓ DOC DATE: 09/10/1999

ASSIGNOR:

✓ SCHREIER, HANS

✓ DOC DATE: 09/22/1999

ASSIGNEE:

ARADIGM CORPORATION
26219 EDEN LANDING ROAD
HAYWARD, CALIFORNIA 94545

SERIAL NUMBER: 09330903 ✓
PATENT NUMBER:

FILING DATE: 06/11/1999 ✓
ISSUE DATE:

MAURICE CARTER, EXAMINER
ASSIGNMENT DIVISION
OFFICE OF PUBLIC RECORDS

DOCKETED

EE/PO
AERX-021

10-18-1999

Form PTO-1595 (Modified)


 101173261
 PATENTS ONLY

 U.S. DEPARTMENT OF COMMERCE
 Patent and Trademark Office

To the Honorable Commissioner of Patents and Trademarks: Please record the attached original documents or copy thereof.

1. Name of conveying party(ies):

 Igor Gonda
 Hans Schreier

 Additional name(s) of conveying party(ies) attached? ☐ Yes ☒ No

3. Nature of conveyance:

- ☒ Assignment (in counterparts)
☐ Merger ☐ Change of Name
☐ Security Agreement
☐ Other:

 Execution Date: September 10, 1999 and
 September 22, 1999

2. Name and address of receiving party(ies):

Name: Aradigm Corporation

Internal Address:

Street Address: 26219 Eden Landing Road

City: Hayward State: California ZIP: 94545

 Additional name(s) & address(es) attached? ☐ Yes ☒ No

4. Application numbers(s) or patent number(s): 09/330,903, filed June 11, 1999

If this document is being filed together with a new application, the execution date of the application is:

A. Patent Application No.(s)

09/330,903

B. Patent No.(s)

5. Name and address of party to whom correspondence concerning document should be mailed:

 Name: Karl Bozicevic
 Street Address: BOZICEVIC, FIELD, & FRANCIS LLP
 285 Hamilton Avenue, Suite 200
 Palo Alto, California 94301

 Telephone: (650) 327-3400
 Facsimile: (650) 327-3231
6. Total number of applications and patents involved: 01

7. Total fee (37 CFR 3.41): \$40.00

- ☒ Enclosed
☐ Authorized to be charged to deposit account

8. Deposit Account No. 50-0815

The Commissioner is authorized to charge any underpayment or credit any overpayment to Deposit Account No. 50-0815 with regards to this correspondence.

10/15/1999 MTHAI1 00000264 09330903

DO NOT USE THIS SPACE

0 FC-581 40.00 GP

9. Statement and signature.

To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the original document.

Paula A. Borden
 Name of Person Signing

 Signature

Oct. 7, 1999
 Date
Total number of pages comprising cover sheet 03

**ASSIGNMENT OF APPLICATION
(JOINT)**

Address to:
Box Assignment
Assistant Commissioner for Patents
Washington, D.C. 20231

Attorney Docket Number	6513-061US1
First Named Inventor	Gonda, et al.
Application Number	09/330,903
Filing Date	June 11, 1999
Title	Methods of Delivering Aerosolized...
Group Art Unit	N/A
Examiner Name	N/A

THIS ASSIGNMENT, by Igor Gonda and Hans Schreier (hereinafter referred to as the assignors), residing at San Francisco, California and Sebastopol, Francisco, California respectively, witnesseth:

WHEREAS, the said assignors have invented certain new and useful improvements in Methods of Delivering Aerosolized Polynucleotides to the Respiratory Tract

 X for which an application for a United States Patent was filed on June 11, 1999, Application No. 09/330,903.

 for which an application for a United States Patent was executed on , and

WHEREAS, Aradigm Corporation a corporation duly organized under and pursuant to the laws of California, and having its principal place of business at 26219 Eden Landing Road, Hayward, California 94545 (hereinafter referred to as the assignee) is desirous of acquiring the entire right, title and interest in and to said invention and said application for Letters Patent of the United States, and in and to any Letters Patent or Patents, United States or foreign, to be obtained therefor and thereon:

NOW THEREFORE, in consideration of One Dollar (\$1.00) and other good and sufficient considerations, the receipt of which is hereby acknowledged, said assignors have sold, assigned, transferred and set over, and by these presents do sell, assign, transfer and set over, unto the assignee, its successors, legal representatives and assigns, the entire right, title and interest in and to the above-mentioned invention, application for Letters Patent, and any and all Letters Patent or Patents in the United States of America and all foreign countries which may be granted therefor and thereon, and in and to any and all divisions, continuations, and continuations-in-part of said application, or reissues or extensions of said Letters Patent or Patents, and all rights under the International Convention for the Protection of Industrial Property, the same to be held and enjoyed by said assignee, for its own use and behoof and the use and behoof of its successors, legal representatives and assigns, to the full end of the term or terms for which Letters Patent or Patents may be granted, as fully and entirely as the same would have been held and enjoyed by the assignors, had this sale and assignment not been made.

AND for the same consideration, said assignors hereby covenant and agree to and with said assignee, its successors, legal representatives and assigns, that, at the time of execution and delivery of these presents, said assignors are the sole and lawful owners of the entire right, title and interest in and to said invention and the application for Letters Patent above-mentioned, and that the same are unencumbered and that said assignors have good and full right and lawful authority to sell and convey the same in the manner herein set forth.

AND for the same consideration, said assignors hereby covenant and agree to and with said assignee, its successors, legal representatives and assigns, that said assignors will, whenever counsel of said assignee, or the counsel of its successors, legal representatives and assigns, shall advise that any proceeding in connection with said invention, or said application for Letters Patent, or any proceeding in connection with Letters Patent for said invention in any country, including interference proceedings, is lawful and desirable, or that any division, continuation or continuation-in-part of any application for Letters Patent or any reissue or extension of any Letters Patent, to be obtained thereon, is lawful and desirable, sign all papers and documents, take all lawful oaths, and do all acts necessary or required to be done for the procurement, maintenance, enforcement and defense of Letters Patent for said invention, without charge to said assignee, its successors, legal representatives and assigns, but at the cost and expense of said assignee, its successors, legal representatives and assigns.

AND said assignors hereby request the Commissioner of Patents to issue said Letters Patent of the United States to said assignee as the assignee of said invention and the Letters Patent to be issued thereon for the sole use and behoof of said assignee, its successors, legal representatives and assigns.

Date September 10, 1999 Name of Inventor

Igor Gonda

Date _____ Name of Inventor

Hans Schreier

**ASSIGNMENT OF APPLICATION
(JOINT)**

Address to:
Box Assignment
Assistant Commissioner for Patents
Washington, D.C. 20231

Attorney Docket Number	6513-061US1
First Named Inventor	Gonda, et al.
Application Number	09/330,903
Filing Date	June 11, 1999
Title	Methods of Delivering Aerosolized...
Group Art Unit	N/A
Examiner Name	N/A

THIS ASSIGNMENT, by Igor Gonda and Hans Schreier (hereinafter referred to as the assignors), residing at San Francisco, California and Sebastopol, Francisco, California respectively, witnesseth:

WHEREAS, the said assignors have invented certain new and useful improvements in Methods of Delivering Aerosolized Polynucleotides to the Respiratory Tract

 X for which an application for a United States Patent was filed on June 11, 1999, Application No. 09/330,903.

 for which an application for a United States Patent was executed on , and

WHEREAS, Aradigm Corporation a corporation duly organized under and pursuant to the laws of California, and having its principal place of business at 26219 Eden Landing Road, Hayward, California 94545 (hereinafter referred to as the assignee) is desirous of acquiring the entire right, title and interest in and to said invention and said application for Letters Patent of the United States, and in and to any Letters Patent or Patents, United States or foreign, to be obtained therefor and thereon:

NOW THEREFORE, in consideration of One Dollar (\$1.00) and other good and sufficient considerations, the receipt of which is hereby acknowledged, said assignors have sold, assigned, transferred and set over, and by these presents do sell, assign, transfer and set over, unto the assignee, its successors, legal representatives and assigns, the entire right, title and interest in and to the above-mentioned invention, application for Letters Patent, and any and all Letters Patent or Patents in the United States of America and all foreign countries which may be granted therefor and thereon, and in and to any and all divisions, continuations, and continuations-in-part of said application, or reissues or extensions of said Letters Patent or Patents, and all rights under the International Convention for the Protection of Industrial Property, the same to be held and enjoyed by said assignee, for its own use and behoof and the use and behoof of its successors, legal representatives and assigns, to the full end of the term or terms for which Letters Patent or Patents may be granted, as fully and entirely as the same would have been held and enjoyed by the assignors, had this sale and assignment not been made.

AND for the same consideration, said assignors hereby covenant and agree to and with said assignee, its successors, legal representatives and assigns, that, at the time of execution and delivery of these presents, said assignors are the sole and lawful owners of the entire right, title and interest in and to said invention and the application for Letters Patent above-mentioned, and that the same are unencumbered and that said assignors have good and full right and lawful authority to sell and convey the same in the manner herein set forth.

AND for the same consideration, said assignors hereby covenant and agree to and with said assignee, its successors, legal representatives and assigns, that said assignors will, whenever counsel of said assignee, or the counsel of its successors, legal representatives and assigns, shall advise that any proceeding in connection with said invention, or said application for Letters Patent, or any proceeding in connection with Letters Patent for said invention in any country, including interference proceedings, is lawful and desirable, or that any division, continuation or continuation-in-part of any application for Letters Patent or any reissue or extension of any Letters Patent, to be obtained thereon, is lawful and desirable, sign all papers and documents, take all lawful oaths, and do all acts necessary or required to be done for the procurement, maintenance, enforcement and defense of Letters Patent for said invention, without charge to said assignee, its successors, legal representatives and assigns, but at the cost and expense of said assignee, its successors, legal representatives and assigns.

AND said assignors hereby request the Commissioner of Patents to issue said Letters Patent of the United States to said assignee as the assignee of said invention and the Letters Patent to be issued thereon for the sole use and behoof of said assignee, its successors, legal representatives and assigns.

Date

Name of Inventor

Igor Gonda

Date 9/22/99

Name of Inventor

Hans Schreier